Suppression of Tumor Growth by Galectin-7 Gene Transfer

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**ABSTRACT**

Galectin-7 is a β-galactoside-binding animal lectin specifically expressed in stratified epithelia. Its expression is inducible by p53 and is down-regulated in squamous cell carcinomas. Other investigators previously showed that galectin-7 is a proapoptotic protein, and we showed that ectopic expression of galectin-7 in HeLa cells renders the cells more sensitive to a variety of apoptotic stimuli. In the present study, we showed that ectopic expression of galectin-7 in the human colon carcinoma cell line DLD-1 also made the cells more sensitive to apoptosis under various conditions. We also found that galectin-7-transfected DLD-1 (DLD-1-Gal7) cells grew significantly more slowly than control transfectants (DLD-1-V) under normal culture conditions in the absence of apoptosis. Moreover, a significantly lower number of colonies were formed from DLD-1-Gal7 cells than from DLD-1-V cells under anchorage-independent cell growth conditions. Most importantly, tumor formation from DLD-1-Gal7 cells was dramatically reduced compared with DLD-1-V cells when these cells were inoculated s.c. into severe combined immunodeficient mice. DLD-1-Gal7 tumors showed a significantly lower proliferation rate than DLD-1-V tumors as determined by in vivo 5-bromo-2′-deoxyuridine incorporation. DLD-1-Gal7 tumors also contained a lower density of blood vessels than DLD-1-V tumors, suggesting that ectopic expression of galectin-7 suppresses angiogenesis. This may partially account for the greater suppressive effect of galectin-7 on tumor growth in vivo than in vitro. Our results show that galectin-7 has a suppressive effect on tumor growth, suggesting that galectin-7 gene transfer or other means of specifically inducing galectin-7 expression may be a new approach for management of cancers.

**INTRODUCTION**

Galectins are a family of β-galactoside-binding animal lectins (1). To date, 14 members have been identified. Diverse biological functions have been shown for each member, and the picture that has emerged is that many of them appear to function in cellular homeostasis through regulation of cell growth, cell cycle, and apoptosis (2–6). The expression of many galectins appears to be significantly altered in cancers (7). It is conceivable that altered expression of these proteins may contribute to neoplastic transformation and tumor progression through regulation of cell growth, cell cycle, and apoptosis.

Galectin-7 is expressed in stratified epithelial cells (8, 9), but its expression is down-regulated in transformed keratinocytes and squamous cell carcinoma cells (8, 10). Its gene was reported to be one of those highly induced by the tumor suppressor p53 in the human colon carcinoma cell line DLD-1, thus designated as p53-induced gene 1 (PIG1), and the protein has been shown to play a role in the proapoptotic function of p53 (11). A squamous cell carcinoma line stably transfected with human galectin-7 cDNA and control vector (pEF1-neo; DLD-1-Gal7 and DLD-1-V, respectively) were generated as reported previously (13). Stable transfectants were selected in medium containing 1 mg/ml G418 (Calbiochem). The G418-resistant cells were stored in a liquid nitrogen freezer and thawed for use in the experiments. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere containing 10% CO2. For the hypoxic condition, cells were placed in an airtight chamber purged with air containing 90% N2/10% CO2 gas, sealed, and incubated at 37°C.

**Apoposis Assay.** Cells were collected by trypsinization, centrifuged, washed with PBS, and stained with FITC-conjugated annexin V (BD Pharmingen) and propidium iodide. Stained cells were analyzed by flow cytometry (Coulter, Miami, FL) as described previously (13). Caspase-3 activity was measured as described previously (14). Briefly, cells were solubilized in lysis buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 50 μM dithiobin] at 37°C for 10 min. Tumor tissues harvested from mice were lysed in ice-cold lysis buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS (pH 7.4)] containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin with a Dounce-type homogenizer (Wheaton Science Products, Millville, NJ). Lysates were centrifuged, and supernatants were collected. Lysates containing 40 μg proteins were incubated with 50 μM caspase-3 substrate, acetyl-aspartyl-glutamyl-valyl-aspartic acid 4-methyl coumaryl-7-amide, in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA at 37°C. The released 7-amino-4-methyl-coumarin was measured using a plate reader with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Caspase-3 activity was calculated as generated 7-amino-4-methyl-coumarin (nmol)/mg protein lystate/min at 37°C.

**Anchorage-Independent Cell Growth.** Anchorage-independent cell growth was determined by analyzing colony formation of cells in soft agar.

**MATERIALS AND METHODS**

**Reagents.** Propidium iodide, p-indonitrotetrazolium violet, 5-bromo-2′-deoxyuridine (Brdu), and cobalt chloride were purchased from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated anti-Brdu monoclonal antibody and rat antimus CD31 monoclonal antibody were from Chemicon (Temecula, CA) and BD PharMingen (San Diego, CA), respectively. 3,3′-Diaminobenzidine substrate kit was from Pierce (Rockford, IL). Acetyl-aspartyl-glutamyl-valyl-aspartic acid α-4-methyl coumaryl-7-amide was from Calbiochem (San Diego, CA).

**Cell Lines and Cultures.** DLD-1 human colon carcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). DLD-1 cells stably transfected with human galectin-7 cDNA and control vector (pEF1-neo; DLD-1-Gal7 and DLD-1-V, respectively) were generated as reported previously (13). Stable transfectants were selected in medium containing 1 mg/ml G418 (Calbiochem). The G418-resistant cells were stored in a liquid nitrogen freezer and thawed for use in the experiments. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere containing 10% CO2. For the hypoxic condition, cells were placed in an airtight chamber purged with air containing 90% N2/10% CO2 gas, sealed, and incubated at 37°C.

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from the skin and connective tissue. The tumor weight was determined in
jection, the mice were sacrificed. Each tumor was harvested and removed
harvested by trypsinization, washed twice in PBS, and adjusted to 5 × 10^6/ml. The cells (10^6 cells in 200 µl PBS/mouse) were injected s.c. into the dorsal flank of 6–8-week-old female nonobese diabetic (NOD)/SCID mice (The Jackson Laboratory, Bar Harbor, ME). The diameter and height of the tumors were measured once a week using a caliper. The tumor numbers of each sample were counted at least four times.

### In Vivo Tumor Growth
Cultured DLD-1-V or DLD-1-Gal7 cells were harvested by trypsinization, washed twice in PBS, and adjusted to 5 × 10^6 cells/ml. The cells (10^6 cells in 200 µl PBS/mouse) were injected s.c. into the dorsal flank of 6–8-week-old female nonobese diabetic (NOD)/SCID mice (The Jackson Laboratory, Bar Harbor, ME). The diameter and height of the tumors were measured once a week using a caliper. The tumor volume was calculated as length × width × height. Four weeks after injection, the mice were sacrificed. Each tumor was harvested and removed from the skin and connective tissue. The tumor weight was determined using an analytical scale.

### Immunohistochemistry
Mice bearing tumors were given BrdU (50 µg/g body weight) i.p. 2 weeks after the inoculation of DLD-1 cells. Two h after the injection, mice were sacrificed, and the tumors were harvested, fixed in 10% buffered formalin, and embedded in paraffin. Four-µm-thick sections were stained with horseradish peroxidase-conjugated anti-BrdU antibody, followed by the addition of the substrate 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin. The number of BrdU-positive nuclei was counted in three or four high-power fields for each section.

To detect blood vessels in tumors, harvested tumors were fixed in zinc fixative (BD Pharmingen) for 24 h and embedded in paraffin. Four-µm-thick sections were reacted with anti-CD31 antibody, further processed using Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin.

### Statistical Analyses
For all of the experiments, Student’s t test was used to determine statistical differences, with the significance set at p < 0.05.

### RESULTS
Galectin-7-Transfected DLD-1 Cells Are More Sensitive to Apoptotic Stimuli
We previously showed that galectin-7-transfected HeLa cells were more sensitive to apoptosis induced by a variety of apoptotic stimuli compared with control transfectants (13). In the same studies, we also showed that DLD-1-Gal7 cells were more sensitive to apoptosis induced by actinomycin D than control transfectants (DLD-1-V). To determine whether galectin-7 overexpression in DLD-1 cells also renders the cells more sensitive to other apoptotic stimuli, we cultured the cells under low serum and hypoxia conditions. Cells treated with actinomycin D were included as a control. As shown in Fig. 1A, higher percentages of DLD-1-Gal7 cells were stained positively by FITC-annexin V than DLD-1-V cells when the cells were cultured in a low serum or hypoxia condition or a combination of low serum and hypoxia conditions, although the differences were not pronounced. In the same experiments, actinomycin D caused a significantly higher percentage of DLD-1-Gal7 cells to be stained positively by FITC-annexin V compared with DLD-1-V cells (Fig. 1B). These results indicate that DLD-1-Gal7 cells are more sensitive to apoptosis induced by actinomycin D than DLD-1-V cells.

![Fig. 1. Stress-induced apoptosis of DLD-1-V and DLD-1-Gal7 cells. DLD-1-V (open bars) and DLD-1-Gal7 (closed bars) cells were cultured under the following conditions: 10% serum/normoxia, 0.1% serum/normoxia, 10% serum/hypoxia, 0.1% serum/hypoxia, and in the presence of 10 µg/ml actinomycin D. A, cells were collected and stained with FITC-conjugated annexin V and propidium iodide, and the stained cells were analyzed by flow cytometer. B, cells were collected, and caspase-3 activity in the cell lysates was measured. The data are the mean ± SD of triplicate experiments. * denotes that there is a significant difference within each pair (paired Student’s t test, p < 0.05).

![Fig. 2. Growth of DLD-1-V and DLD-1-Gal7 cells in vitro. One hundred thousand DLD-1-V (○) and DLD-1-Gal7 (■) cells were cultured in DMEM containing 10% fetal bovine serum in a 10-cm dish. After indicated time of culture, cells were trypsinized, and the number of viable cells was counted. The data are the mean ± SD of three samples. The cell numbers of each sample were counted at least four times.

![Fig. 3. Anchorage-independent cell growth in soft agar. Three thousand DLD-1-V or DLD-1-Gal7 cells in DMEM containing 0.3% agar and 10% fetal bovine serum were plated onto solidified 0.6% agar in each well of a six-well plate. After 21 days of culture, the cells were stained with 1 mg/ml p-indonitrotetrazolium violet for 24 h, and colonies of >0.1 mm in size were counted. A, photographs of the representative wells of DLD-1-V and DLD-1-Gal7 cells. B, the colonies in five wells of each of DLD-1-V and DLD-1-Gal7 cells were counted. The data are the mean ± SD of number of colonies. The results are representative of two independent experiments.

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with DLD-1-V, as we have shown previously. Similar results were obtained when the cells were treated with two other apoptotic stimuli: cobalt chloride and hydrogen peroxide (data not shown).

We also measured the caspase-3 activity in the treated cells as another means to quantify apoptosis. As shown in Fig. 1B, caspase-3 activity was not detectable in the cells cultured under normoxia condition. In contrast, it was clearly detectable in the cells cultured in hypoxia or treated with actinomycin D. Under both conditions, significantly higher caspase-3 activity was detected in DLD-1-Gal7 cells compared with DLD-1-V cells. The results confirm that galectin-7 overexpression makes DLD-1 cells more sensitive to apoptosis.

Galectin-7-Transfected DLD-1 Cells Grow More Slowly than Control Transfectants. We next examined whether there is any difference in the growth rate in vitro between DLD-1-Gal7 and DLD-1-V cells. As shown in Fig. 2, the growth rate of DLD-1-Gal7 cells was slower than that of DLD-1-V cells under normal culture conditions. Under these conditions, no appreciable apoptosis occurred because caspase-3 activity was not detectable (see Fig. 1B, normoxia).

We also compared the growth rates of these two cell types under anchorage-independent cell growth conditions. After 3 weeks of culture in soft agar, the number of DLD-1-Gal7 colonies was significantly lower than that of DLD-1-V colonies (P < 0.001; Fig. 3, A and B). Therefore, DLD-1-Gal7 cells grow more slowly under two different conditions, in normal adherent culture and in soft agar, the latter being an in vitro model of tumorigenesis.

Galectin-7-Transfected DLD-1 Cells Exhibit Significantly Reduced Growth Rate in Vivo in SCID Mice. We hypothesized that ectopic expression of galectin-7 in cancer cells would result in suppression of tumor growth because of increased apoptosis and decreased growth rate. Other investigators previously showed that...
DLD-1 cells produce tumors when inoculated into immunodeficient mice (15). Thus, we inoculated SCID mice s.c. with DLD-1-Gal7 cells and DLD-1-V cells and monitored the tumor growth. Although DLD-1-V cells grew into tumors as expected, DLD-1-Gal7 cells hardly grew (Fig. 4A). As shown in Fig. 4B, the growth suppression of galectin-7 transfectants was noted during the 4-week period of observation. The striking difference in the tumor formation between the two cell types was reflected when the volumes (Fig. 4C) and weights (Fig. 4D) of the tumors were compared. The growth of DLD-1-V cells was almost the same as the parental cells (data not shown).

**Galectin-7-Transfected DLD-1 Cells Show Reduced Cell Proliferation in Vivo.** The *in vitro* results suggest that the lack of *in vivo* tumor growth of DLD-1-Gal7 cells could be caused by increased apoptosis, decreased growth rate, or both. We measured the caspase-3 activity in the tumors and found that DLD-1-V and DLD-1-Gal7 tumors contained little caspase-3 activity (Fig. 5A). We then measured the number of proliferating cells in each tumor by labeling the tumors with BrdU *in vivo*. The number of BrdU-positive cells per field in DLD-1-Gal7 tumors was much lower than that in DLD-1-V tumors, indicating that fewer cells were proliferating in DLD-1-Gal7 tumors than in DLD-1-V tumors (Fig. 5B). The difference in the number of BrdU-positive cells between two groups was statistically significant (*P* < 0.000001; Fig. 5C).

**Galectin-7-Transfected DLD-1 Tumors Show a Reduced Number of Blood Vessels.** Our data suggested that the growth-inhibitory effect of galectin-7 was greater *in vivo* than *in vitro*. Because angiogenesis is one of the *in vivo* factors that affect the tumor growth, we stained blood vessels in the tumors. When tumors of similar size were compared (DLD-1-V and DLD-1-Gal7 tumors were harvested after 2 and 4–5 weeks, respectively), the density of CD31-positive blood vessels was significantly lower in DLD-1-Gal7 tumors (Fig. 6; *P* < 0.01).

**DISCUSSION**

We previously have shown that ectopic expression of galectin-7 in HeLa cells renders the cells more sensitive to apoptosis induced by a number of different apoptotic stimuli operating through distinct mechanisms (13). Herein, we showed that ectopic expression of another cell line, DLD-1, also results in the cells becoming more sensitive to a number of apoptotic stimuli (Fig. 1). The results suggest that galectin-7 is proapoptotic in a number of different cell types. Furthermore, our studies showed that galectin-7 is a negative regulator of cell growth (Fig. 2). Most importantly, galectin-7 overexpression results in a profound retardation of the growth of DLD-1 cells *in vivo* in SCID mice (Fig. 4).

Our initial hypothesis was that galectin-7 transfectants transplanted onto mice would be more sensitive to chemotherapeutic agents *in vivo*. However, we found that these transfectants barely grew even in the absence of these cytotoxic agents. It is possible that galectin-7 transfectants are more sensitive to apoptosis induced by the stressful conditions caused by the low nutrients or hypoxia in the microenvironment (Fig. 1).

However, using caspase-3 activity assay we found that there were comparably low numbers of apoptotic cells in DLD-1-Gal7 and DLD-1-V cells grown *in vivo* (Fig. 5A). Thus, it appears that apoptosis does not appreciably account for the decreased growth of transplanted DLD-1-Gal7 cells. One remaining possibility is that more apoptotic cell death occurs in DLD-1-Gal7 cells during the early period after the transfectants are inoculated to SCID mice. This might not be detectable at the time the tissues were taken for the apoptosis assay.

On the other hand, our studies revealed a new function of galectin-7 as a negative regulator of cell growth. DLD-1-Gal7 cells grew significantly more slowly than DLD-1-V cells under normal and stressful (like low serum or hypoxia) growth conditions (Fig. 2 and data not shown). The former also showed a reduced growth rate under the anchorage-independent cell growth condition (Fig. 3). This function probably contributes at least in part to the retarded cell growth of...
galectin-7 transfectants in vivo. This is confirmed by the lower incorporation of BrdU into DLD-1-Gal7 cells compared with DLD-1-V cells transplanted onto SCID mice (Fig. 5).

Immunostaining of DLD-1-Gal7 cells showed that galectin-7 mainly is present in the cytosol and the nucleus, but little is found on the membrane (data not shown), suggesting that the growth-inhibitory effect in DLD-1-Gal7 tumors mainly is produced by intracellular galectin-7 (13). However, it recently was reported that exogenous galectin-7 reduces the proliferation of cancer cells through its binding to glycolipids of the cell membrane (16). A recent report also described that lower amounts of galectin-1 can induce cell death when it exists on the cell membrane or in the extracellular matrix (17). Therefore, we cannot completely exclude the possibility that galectin-7 can function extracellularly by a juxtacrine mode, and additional studies will be required to address this issue.

Our data showed that the growth-inhibitory effect of galectin-7 was greater in vivo than in vitro. The results suggest that some other in vivo factors not discussed previously may contribute to the overall growth-suppressive effect of galectin-7. Factors affecting angiogenesis could be an example because angiogenesis is required for s.c. inoculated tumors to grow beyond the microscopic size (18, 19). The histologic analysis by CD31 staining showed that the density of CD31-positive blood vessels was significantly lower in DLD-1-Gal7 tumors (Fig. 6). It was reported that DLD-1 cells with reduced expression of vascular endothelial growth factor (VEGF) exhibit profoundly suppressed growth in nude mice (20). However, there was little difference in the levels of VEGF mRNA and VEGF protein secreted into the culture medium between DLD-1-V and DLD-1-Gal7 cells under normal culture conditions and conditions that mimic hypoxia (i.e., in the presence of cobalt chloride; data not shown). Additional studies are required to determine the involvement of angiogenic factor(s) other than VEGF.

In summary, we showed here that galectin-7 inhibits cell growth not only in vitro but also in vivo. The inhibitory effect of galectin-7 on the tumor growth in vivo is attributable to the suppression of cell growth and the reduced ability of the anchorage-independent growth rather than increased sensitivity of apoptosis. Reduced angiogenesis may also contribute to the tumor-suppressive effect of galectin-7. p53 gene transfer has long been actively pursued as a universal approach for management of different types of cancer based on its effects in suppressing cell growth and inducing apoptosis. Many p53-targeted genes have been reported, including galectin-7; some (e.g., p21 and 14–3–3-σ; Refs. 21–23) induce only cell cycle arrest, and others induce only apoptosis (e.g., Bax, PIG3, PUMA; Refs. 11, 24–26). Our data show that similar to p53, galectin-7 suppresses cell growth and increases sensitivity to apoptosis. We conclude that ectopic expression of galectin-7 in cancers, either through gene transfer or specific induction of gene expression, may be a novel modality of cancer therapy, and galectin-7 gene therapy may be applicable to all of the types of cancers.

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